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## Titanium ions form particles that activate and execute interleukin-1 release from lipopolysaccharide-primed macrophages

Pettersson, M ; Kelk, P ; Belibasakis, Georgios N ; Bylund, D ; Molin Thorén, M ; Johansson, A

**Abstract:** BACKGROUND AND OBJECTIVE: Peri-implantitis is a destructive inflammatory process characterized by destruction of the implant-supporting bone. Inflammasomes are large intracellular multi-protein complexes that play a central role in innate immunity by activating the release of proinflammatory cytokines. Although inflammasome activation has previously been linked to periodontal inflammation, there is still no information on a potential association with peri-implantitis. The aim of this study was to examine cytotoxic and proinflammatory effects, including inflammasome activation, of metals used in dental implants, in an in vitro model, as well as from clinical tissue samples. MATERIAL AND METHODS: Human macrophages were exposed to different metals [titanium (Ti), cobalt, chromium and molybdenum] in a cell-culture assay. Cytotoxicity was determined using the neutral red uptake assay. Cytokine secretion was quantified using an ELISA, and the expression of genes of various inflammasome components was analysed using quantitative PCR. In addition, the concentrations of interleukin-1 (IL-1) and Ti in mucosal tissue samples taken in the vicinity of dental implants were determined using ELISA and inductively coupled plasma mass spectrometry, respectively. RESULTS: Ti ions in physiological solutions stimulated inflammasome activation in human macrophages and consequently IL-1 release. This effect was further enhanced by macrophages that have been exposed to lipopolysaccharides. The proinflammatory activation caused by Ti ions disappeared after filtration (0.22 µm), which indicates an effect of particles. Ti ions alone did not stimulate transcription of the inflammasome components. The Ti levels of tissue samples obtained in the vicinity of Ti implants were sufficiently high (> 40 µg/g) to stimulate secretion of IL-1 from human macrophages in vitro. CONCLUSION: Ti ions form particles that act as secondary stimuli for a proinflammatory reaction.

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# Titanium ions form particles that activate and execute interleukin-1 $\beta$ release from lipopolysaccharide-primed macrophages

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**Background and Objective:** Peri-implantitis is a destructive inflammatory process characterized by destruction of the implant-supporting bone. Inflammasomes are large intracellular multiprotein complexes that play a central role in innate immunity by activating the release of proinflammatory cytokines. Although inflammasome activation has previously been linked to periodontal inflammation, there is still no information on a potential association with peri-implantitis. The aim of this study was to examine cytotoxic and proinflammatory effects, including inflammasome activation, of metals used in dental implants, in an *in vitro* model, as well as from clinical tissue samples.

**Material and methods:** Human macrophages were exposed to different metals [titanium (Ti), cobalt, chromium and molybdenum] in a cell-culture assay. Cytotoxicity was determined using the neutral red uptake assay. Cytokine secretion was quantified using an ELISA, and the expression of genes of various inflammasome components was analysed using quantitative PCR. In addition, the concentrations of interleukin-1 $\beta$  (IL-1 $\beta$ ) and Ti in mucosal tissue samples taken in the vicinity of dental implants were determined using ELISA and inductively coupled plasma mass spectrometry, respectively.

**Results:** Ti ions in physiological solutions stimulated inflammasome activation in human macrophages and consequently IL-1 $\beta$  release. This effect was further enhanced by macrophages that have been exposed to lipopolysaccharides. The proinflammatory activation caused by Ti ions disappeared after filtration (0.22  $\mu$ m), which indicates an effect of particles. Ti ions alone did not stimulate transcription of the inflammasome components. The Ti levels of tissue samples obtained in the vicinity of Ti implants were sufficiently high ( $\geq 40 \mu$ M) to stimulate secretion of IL-1 $\beta$  from human macrophages *in vitro*.

**Conclusion:** Ti ions form particles that act as secondary stimuli for a proinflammatory reaction.

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**Key words:** caspase-1; inflammation; interleukin-1 $\beta$ ; macrophage; peri-implantitis; titanium

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Hundreds of thousands of patients worldwide are treated with dental implants each year (1,2). Dental implants were first introduced by Brånemark in the 1960s and are currently the standard treatment for single tooth loss and for partially and total edentulous patients (3–5). The treatment is most reliable for restoring edentulism, with reported success rates of more than 90% (6–9). However, in recent years reports have shown increasing numbers of patients with peri-implantitis, resulting in bone loss around implants. The reported prevalence of peri-implantitis ranges from 11.2% to 53% for subjects and from 4.7% to 36.6% for implants (10–18). The substantial variability of the estimated prevalence of peri-implantitis reported in the literature indicates that either there is a true difference in different cohorts of patients or the true prevalence of peri-implantitis is not yet known. One of the reasons for this large discrepancy is the inconsistent criteria in the definition of peri-implantitis.

At the first European Workshop on Periodontology in 1993 it was agreed that the term peri-implantitis should be used specifically for destructive inflammatory processes around osseointegrated implants in function that lead to peri-implant 'lesion' formation and the loss of supporting bone (19).

It is hypothesized that the inflammatory response around osseointegrated implants is an immune response produced against oral bacteria to prevent them establishing a biofilm on the implant surface (18,20,21). The immunopathological events that govern peri-implantitis resemble those of periodontitis, yet they are considerably more pronounced (22). The biofilm microbiota associated with peri-implantitis shows strong similarities to periodontitis, yet recent metagenomic analyses may also reveal some distinctive differences between the two (23). Of special note regarding the pathogenesis of peri-implantitis, Albrektsson *et al.* (24) propose that it largely accounts for an immunological foreign-body response to titanium, a hypothesis which awaits further investigation.

The definition of peri-implantitis by Albrektsson & Isidor (19) is still used, but there is no clear distinction between slight, moderate and severe peri-implantitis. Severe periodontitis is reported to have a global prevalence of 11% on global basis (25,26) and probably peri-implantitis will show about the same prevalence as more research data become published in the literature. Atieh *et al.* (27) reported, in a review based on 504 identified studies, estimates for the frequency of peri-implant mucositis of 63.4% for participants and 30.7% for implants and for the frequency of peri-implantitis of 18.8% for participants and 9.6% for implants. An even higher frequency of the occurrence of peri-implant diseases was recorded for smokers, with a summary estimate of 36.3%. These prevalence numbers show that peri-implant mucositis and peri-implantitis are not uncommon in patients undergoing dental implant treatment and should not be neglected, especially in high-risk patients.

In comparison with orthopedic implants that are placed in a sterile environment and accordingly should not be exposed to bacteria or bacterial products, dental implants are exposed to the environment of the mouth. However, orthopedic implants induce an inflammatory response, similar to that of peri-implantitis, around dental implants, and loss of orthopedic implants are described in the literature as aseptic loosening, first reported by Harris *et al.* (28). Aseptic loosening of an orthopedic prosthesis is one of the most frequent complications leading to a revision of the prosthesis, and much research has been carried out during the last 10 years to identify the mechanism of the peri-prosthetic osteolysis leading to failure of the prosthesis (29–32).

The alarm cytokine, interleukin (IL)-1 $\beta$ , possesses a wide spectrum of inflammatory, metabolic, physiologic, hematopoietic and immunologic properties and plays a crucial role in the innate immune system (33). When the toll-like receptors on macrophages are activated by pathogen-associated molecular patterns, production of pro-IL-1 $\beta$  in cytoplasm is initiated

and upon a secondary stimulation that involves inflammasome activation, finally active IL-1 $\beta$  can be processed and secreted (34–36). Release of active IL-1 $\beta$  initiates inflammation, leading to infiltration of leukocytes to eliminate the infectious agents (37). IL-1 $\beta$  is perhaps one of the most potent cytokines, causing inflammation and fever, and also induces expression of proinflammatory genes that encode IL-6 and nitric oxide synthase, and increases expression of adhesion molecules, promoting leukocyte migration from the bloodstream into the infected tissue (37–39).

Activation and release of IL-1 $\beta$  is mediated by the assembly of a cytosolic multimer consisting of NLR family, pyrin domain containing 3 (NLRP3), apoptosis-associated Speck-like Protein Containing a Card (ASC) and pro-caspase-1, named the NLRP3 inflammasome (40). Activation of the NLRP3 inflammasome results in active caspase-1 that stimulates release of mature IL-1 $\beta$  from macrophages would be more accurate, which can occur from both pathogen-associated molecular patterns and damage-associated molecular patterns (40,41). It has been shown that different compounds, such as flagellin, double-stranded DNA and various crystals (asbestos, alum, silica, cholesterol and uric acid), can mediate activation and release of mature IL-1 $\beta$  (42–48). Moreover, a newly discovered mechanism of cell death, termed pyroptosis, leads to activation specifically of caspase-1 (49). The term pyroptosis is now accepted as a death mechanism, and macrophages undergoing pyroptosis show signs of both apoptosis and necrosis with specific activation of caspase-1 (50). Caspase-1 is responsible for activation of pro-IL-1 $\beta$ , pro-IL-18 and pro-IL-33 to their active forms and secretion (51,52). Several bacterial products, such as lipopolysaccharide (LPS), are known to increase expression of pro-IL-1 $\beta$  in macrophages (53). However, a secondary stimulus is needed to induce secretion of a high level of bioactive IL-1 $\beta$ . This activation can be induced by various bacterial species through activation of the inflammasome complex, caspase-1 acti-

vation and ultimately IL-1 $\beta$  secretion (54). This secondary activation can be induced by direct interactions of bacteria or bacterial components with the inflammasome complex (such as for *Salmonella typhimurium* or *Francisella tularensis*) or through cell-surface interactions (such as for *Listeria monocytogenes*, *Staphylococcus aureus*, *Aggregatibacter actinomycetemcomitans* or *Porphyromonas gingivalis*) (54–60). Oral biofilms are also shown to regulate expression of the inflammasome complexes (61), and expression of NLRP3 inflammasome in gingival tissue is shown to be increased in periodontal disease, compared to healthy periodontal conditions (59).

Caicedo *et al.* (62) showed that soluble and particulate forms of cobalt–chromium–molybdenum (Co–Cr–Mo) alloys activate the NLR family, pyrin domain containing 3 inflammasome danger-signaling pathway in human macrophages, leading to secretion of IL-1 $\beta$ . Recently published data suggest that sodium fluoride corrodes titanium (Ti) implants, and the release of Ti ions is suggested to activate factors of importance for bone resorption (63). As previously shown by our group, rapid degradation of the tooth-supporting tissues in young individuals is associated with the presence of highly leukotoxic *A. actinomycetemcomitans* (64). The leukotoxin produced by this bacterium activates the inflammasome complex and caspase-1 in human macrophages, and consequently stimulates activation and release of IL-1 $\beta$  (56). *Aggregatibacter actinomycetemcomitans* is also shown to activate the expression of NLRP3 and down-regulate the expression of NLRP6 in human mononuclear cells (60).

In the present study, we hypothesize that materials released from dental implants and overlaying dental constructions contribute to the inflammatory reactions associated with peri-implantitis by stimulating inflammasome activation and IL-1 $\beta$  secretion in macrophages. We show that Ti ions form particles in a physiological solution and induce a proinflammatory reaction in human macrophages *in vitro*. In addition, this activation is

caused by the increased Ti concentration that could be detected in samples of tissue taken in close vicinity to dental implants.

## Material and methods

### Growth medium and cell culture

A human acute monocytic leukemia cell line THP-1 (ATCC<sup>®</sup> TIB-202<sup>™</sup>) was purchased from the American Type Culture Collection (ATCC<sup>®</sup>; Manassas, VA, USA) and cultured in RPMI 1640 containing 10% fetal bovine serum (FBS) with a supplement of penicillin–streptomycin (Sigma-Aldrich; St Louis, MO, USA). The THP-1 cell line originates from an anonymous 1-year-old infant boy with acute monocytic leukemia.

Buffy coats (enriched leukocyte fraction) were obtained from heparinized venous blood, taken from healthy donors at Norrlands University Hospital blood bank (Umeå, Sweden). Informed written approval was given by all subjects, and authorization for the study was granted by the Human Studies Ethical Committee of Umeå University, Sweden (§67/3, dnr 03–019). Mononuclear lymphocytes were isolated from buffy coat with isopycnic centrifugation in Lymphoprep<sup>™</sup> (Axis-Shield, Oslo, Norway), as first described by Boyum (65). The mononuclear leukocyte-containing fraction was collected, centrifuged at 220 *g* for 5 min and washed in phosphate-buffered saline three times to remove platelets.

The cell pellet was resuspended in the growth medium RPMI 1640, containing 10% FBS with a supplement of penicillin–streptomycin (Sigma-Aldrich), to a cell concentration of  $5 \times 10^6$  cells/mL.

The suspension was distributed into a 24-well culture plate (Nunc A/S, Roskilde, Denmark), at 1 mL/well, and was incubated at 37°C under 5% CO<sub>2</sub> for 2 h to allow the monocytes to adhere. The nonadherent lymphocytes were removed by two rinses with 1 mL of phosphate-buffered saline. The culture plate was put on ice to detach adherent cells, which were thereafter harvested by centrifugation (220 *g* for 5 min) and resuspended in growth

medium to a concentration of  $4 \times 10^6$  cells/mL. The adherent cells consisted of approximately 95% monocytes, in a total number of about  $10^6$  cells/well.

### Stimulation agents

Plasma standard solution, Specpure<sup>®</sup>, 1000  $\mu$ g/mL, for Co, Cr, Ti and Mo, was purchased from Alfa Aesar GmbH & Co.KG (Karlsruhe, Germany). Plasma standards have a content of acid to stabilize the metal in an ionic form, Cr in 5% HCl for Cr; 5% nitric acid (HNO<sub>3</sub>) for Co; and 5% HNO<sub>3</sub>/trace (tr). hydrogen fluoride (HF) for Ti and Mo.

Measurement of acidity was determined with a pH meter (Beckman), and ion solutions of Co, Cr, Ti and Mo were adjusted to pH 7.2 with 1 M NaOH and diluted to a concentration of 1600  $\mu$ M in RPMI 1640.

### Filtration

Filtration of soluble Co, Cr, Ti and Mo was carried out through a 0.22- $\mu$ m sterile filter (Merck Millipore, Billerica, MA, USA). A solution of 1000  $\mu$ M in cell-growth medium RPMI 1640 + 10% FBS was mixed and divided into three groups, as follows: unfiltered; filtered after 1 h; and filtered after 24 h. Samples of the test solutions were sent to ALS Scandinavia AB, Luleå, Sweden, for measurement of the Ti content.

After the pilot test with filtering of the solutions, it was decided to only test filtration of Ti for cell stimulation. The Ti solution was divided in two groups: one group was unfiltered with a concentration range of 0–400  $\mu$ M; and in the other group the solution was filtered through a 0.22- $\mu$ m sterile filter (Merck Millipore) to determine whether Ti ions suspended in RPMI 1640 form particles. THP-1 cells were then exposed to the filtered and unfiltered Ti solutions, according to the protocol for cell stimulation described above.

### Clinical samples

Three patients, treated 5 years previously with a fixed full-arch implant bridge supported by six Nobel Bio-



care (Zürich, Switzerland) Mark III regular platform Brånemark implants, were randomly selected consecutively by their surname.

A clinical examination was performed and all implants were evaluated and graded as healthy/peri-implant mucositis/peri-implantitis, according to the classification by Albrektsson and Isidor (19). Two deep soft-tissue biopsies were taken from each patient in close vicinity to the implant. The biopsy sites were selected, after clinical examination, at the clinically healthiest implant and at an implant showing signs of inflammation. Samples of the crevicular fluid were collected from the peri-implant pocket at the biopsy sites, before taking biopsy samples, for IL-1 $\beta$  quantification, as described by Gamonal *et al.* (66). The sample areas were isolated with cotton rolls and gently dried with an air syringe before collection of the peri-implant crevicular fluid (PICF) to avoid contamination from saliva. A standard filter paper strip was used as absorbent and gently inserted into the peri-implant pocket and left for 30 s to collect the PICF sample. After collection of the PICF, the strip was put into a sterile Eppendorf tube and sent to the laboratory for analysis within 1 h and the protein was eluted as described by Kelk *et al.* (57). After collection of PICF, a biofilm sample was taken from the same sites by inserting a sterile paper point into the peri-implant pocket for 30 s. After collection, the paper point was placed into a viability-preserving microbiostatic, anaerobic (VMGAIII) transport medium supplemented with Nystatin (2 mg/L), as described earlier (67). Ethical approval (Dnr 2011-405-31M) for the biopsies and biological samples from patients were obtained from the Regional Ethical Review Board at Umeå University, Sweden.

#### Cell stimulation

One-hundred microliters of THP-1 cells suspended in RPMI 1640 were distributed in a 96-well culture plate at a cell concentration of  $10^6$  cells/mL. Phorbol 12-myristate 13-acetate (Sigma-Aldrich) was added to a concentration of 50 nM, and the cells were

incubated at 37°C under 5% CO<sub>2</sub> for 24 h, to differentiate THP-1 cells toward the macrophage phenotype.

Lipopolysaccharides from *Escherichia coli* (*E. coli* LPS) were purchased from Sigma-Aldrich. After 24 h of culture at 37°C under 5% CO<sub>2</sub>, THP-1 cells were primed with 100 ng/mL of *E. coli* LPS for 6 h. Growth medium was then replaced with 100  $\mu$ L of RPMI 1640 containing the stimulatory agents, Co, Cr, Ti or Mo at the highest concentration (800  $\mu$ M) and serially diluted to concentrations of 400, 200, 100, 50, 25, 12.5, 6.3, 3.1 and 0  $\mu$ M. Cells were exposed to these agents for 24 h and then the experiment was terminated. To determine if stimulation with the metals activates secretion of IL-1 $\beta$  through activation of the NLRP3 inflammasome, a known caspase-1 inhibitor, Ac-YVAD-CMK (Calbiochem Merck KGaA, Darmstadt, Germany), was added in some of the *in vitro* experiments with THP-1 cells.

#### RNA extraction and cDNA synthesis

Once the exposure period was complete, the cell culture supernatants were removed by centrifugation, whereas the cell pellets were washed twice in phosphate-buffered saline and lysed. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. The RNA concentration was measured spectrophotometrically, and 400 ng of total RNA was reverse transcribed into cDNA by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in a volume of 20  $\mu$ L. The resulting cDNA was stored at -20°C until further use.

#### Quantitative real-time polymerase chain reaction

For gene-expression analyses, quantitative real-time PCR (qPCR) was performed using a Step One Plus Real Time PCR System (Applied Biosystems). For the amplification reactions, the TaqMan Gene Expression Master Mix and Gene Expression Assay kits from Applied Biosystems were used (assay IDs: *NLRP3*: Hs00918085-m1;

*ASC*: Hs01547324-m1; *CASP1*: Hs00354836-m1; *IL1 $\beta$* : Hs00174097-m1; and *GAPDH*: Hs99999905-m1). The standard PCR conditions were 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The expression levels of the target transcripts in each sample were calculated by the comparative cycle threshold ( $C_t$ ) method ( $2^{-\Delta C_t}$  formula) after normalization to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) housekeeping gene.

#### Cytotoxicity

*In vitro* cytotoxicity of the stimulatory agents on THP-1 cells at the concentrations tested was measured using the neutral red uptake assay, following the protocol described by Repetto *et al.* (68). The neutral red uptake assay is a simple technique used to quantify the proportion of viable cells in culture by addition of the dye neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) (Sigma-Aldrich), which is incorporated and accumulated in the lysosomes of viable cells. After 24 h of exposure of THP-1 cells to stimulants, the supernatant was removed and the neutral red assay was performed on the cells. The absorbance of the extracted neutral red from the challenged cells was read in a spectrophotometer at a wavelength at 540 nm, using the blank without cells as the reference.

#### Cytokine analyses with ELISA

A "sandwich" ELISA (the Human IL-1 $\beta$  DuoSet ELISA; R&D Systems, Inc. Minneapolis, MN, USA) was used to analyze the cell culture supernatant to detect IL-1 $\beta$  secretion by THP-1 cells and human primary monocytes after stimulation with *E. coli* LPS and metals. After 24 h of exposure of the cells, the supernatant was removed and the ELISA was performed following the protocol provided by the manufacturer. A spectrophotometer measuring a wavelength of 450 nm was used to quantify the amount of IL-1 $\beta$  secreted into the supernatant. A human multi-analyte ELISA Array from SABiosciences Corporation (Frederick, MD,

USA) was performed as a screen on primary human monocytes exposed to Ti ions. Cytokines analyzed in the screening were IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17 $\alpha$ , interferon gamma, tumor necrosis factor alpha and granulocyte-macrophage colony-stimulating factor.

### Inductively coupled plasma mass spectrometry

Inductively coupled plasma mass spectrometry was first introduced by Houk *et al.* (69) and first commercialized by PerkinElmer (Waltham, MA, USA) in 1983. Samples were prepared by mixing approximately 0.02 g of lyophilized tissue material with 2 mL of nitric acid for trace analysis (Sigma Aldrich) in high-pressure reaction vessels with Teflon micro sampling inserts. Digestion of the samples was then performed on an Ethos 1 microwave digestion system (Milestone, Sorisole, Italy) employing a microwave program reaching 180°C within 5 min and then held at 175–185°C for 10 min. The resulting clear solutions were transferred into acid-washed sample tubes together with 4 mL of HNO<sub>3</sub> that was used to rinse the inserts. The samples were then diluted 10 times with ultrapure Milli-Q water (Millipore) before the elemental analysis was conducted on a 7700 Series ICP-MS system (Agilent Technologies; Santa Clara, CA, USA) operated in both the no gas mode and the helium (He) mode using indium as an internal standard added online. The instrument was calibrated with Ti standard solutions prepared from a 1000  $\mu$ g/mL Ti standard (Ultra Scientific; North Kingstown, RI, USA) and 2% HNO<sub>3</sub> in Milli-Q water within the range 100–2000 ng/L. The Ti concentrations measured were adjusted for the average concentration measured for blanks prepared in the same way as the samples, but with no biological material added, and the elemental content was finally reported as  $\mu$ M in tissue.

### Statistical analysis

The statistical significance of differences was evaluated using one-way

ANOVA and two-way ANOVA, using Sidak correction and Dunnett's test for multiple comparisons. The data were considered significant at  $p \leq 0.05$ .

## Results

### Proinflammatory activation in cultures of primary monocytes

The proinflammatory effect of Ti was studied in cultures of primary monocytes isolated from peripheral blood. A multi-analyte ELISA was employed to screening the effects on the cytokines IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17 $\alpha$ , interferon- $\gamma$ , tumor necrosis factor alpha and granulocyte-macrophage colony-stimulating factor. The monocyte cultures were primed for 6 h, with or without *E. coli* LPS (100 ng/mL), and exposed to Ti (25  $\mu$ M) for 24 h. This screening experiment was performed once and analyzed in duplicate, in accordance with the manufacturer's protocol (SABiosciences Corporation). The effect of the metal alone on the secretion of all the cytokines studied was limited, while LPS priming enhanced secretion of IL-1 $\beta$ , IL-6, IL-8, IL-10,

interferon- $\gamma$  and granulocyte-macrophage colony-stimulating factor (Fig. 1). The presence of the metal enhanced the secretion of IL-1 $\beta$  in the monocyte cultures that were primed with *E. coli* LPS. The effect of different metals (Co, Cr, Ti and Mo) at low concentrations ( $\leq 25$   $\mu$ M) on the secretion of IL-1 $\beta$  from primary monocytes was further estimated using conventional ELISA. The results obtained after 24 h of exposure of the monocytes to the different metals, with or without priming with *E. coli* LPS (6 h, 100 ng/mL), further confirmed that the effect on IL-1 $\beta$  secretion of the metals alone was limited, whereas an enhanced effect was documented in combination with LPS priming of the target cells (Fig. 2A–D). Ti acted as a secondary stimulus in cultures of LPS-primed (primary stimuli) human macrophages, resulting in activation and secretion of IL-1 $\beta$ .

### Gene-expression analyses of inflammasome components

The effects of the different metals (Co, Cr, Ti and Mo) on gene expression of the inflammasome components

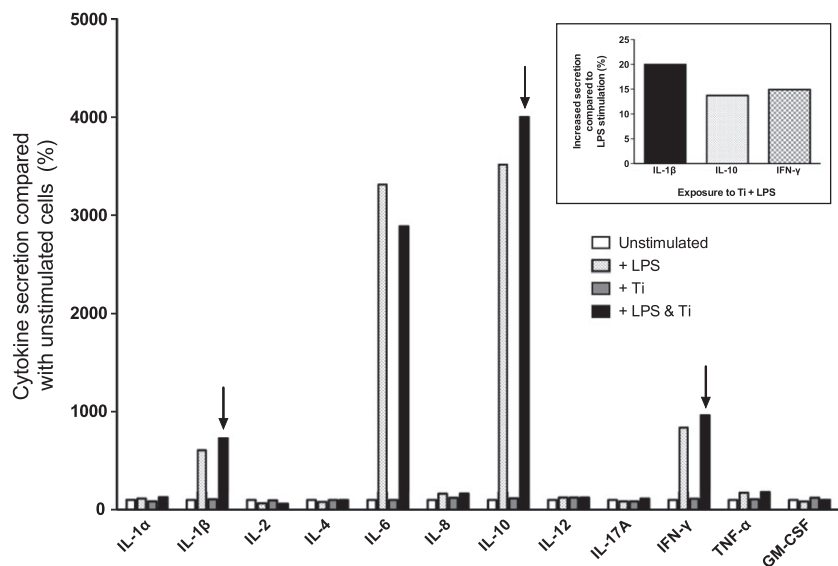


Fig. 1. Screening test with multi-analyte data of cytokine secretion from macrophages isolated from peripheral blood and exposed to titanium (Ti) (for 24 h), with or without priming with *Escherichia coli* lipopolysaccharide (LPS) (6 h). Mean results of duplicates from one experiment are shown. GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ . Arrows indicate enhanced values caused by Ti shown in the inserted diagram.

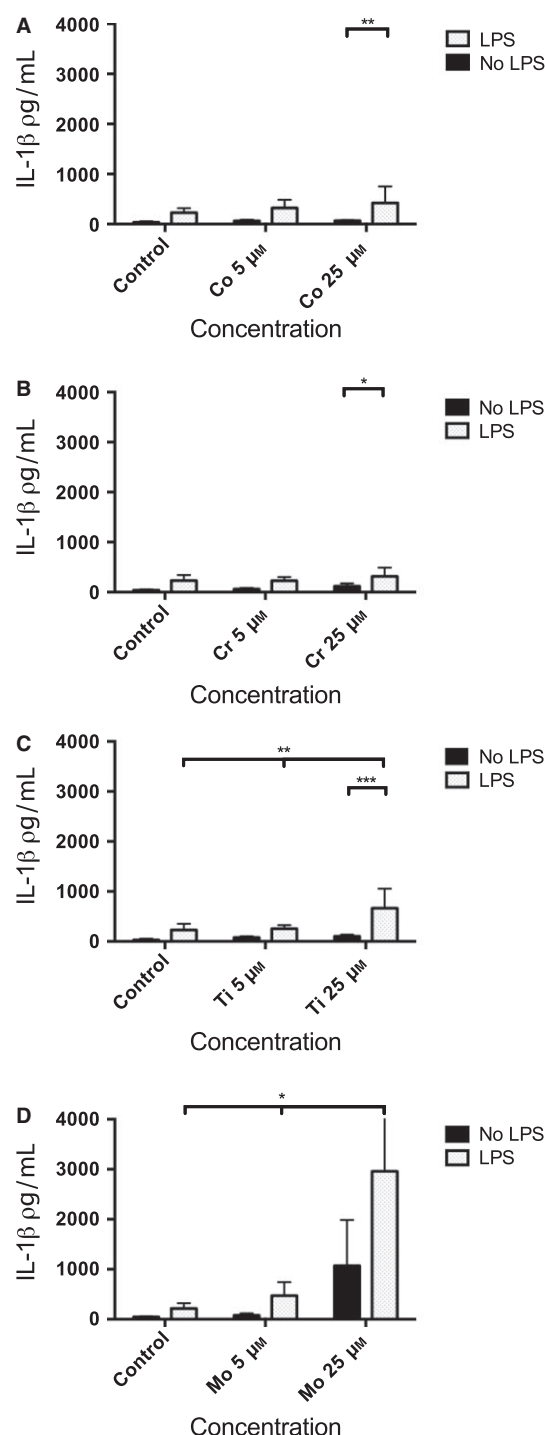


Fig. 2. Secretion of interleukin (IL)-1 $\beta$  from macrophages isolated from peripheral blood and exposed to solutions of 0, 5 or 25  $\mu$ M of cobalt (Co), chromium (Cr), titanium (Ti) or molybdenum (Mo) for 24 h, with and without priming with *Escherichia coli* lipopolysaccharide (LPS) for 6 h. (A) Macrophages exposed to Co solution. (B) Macrophages exposed to Cr solution. (C) Macrophages exposed to Ti solution. (D) Macrophages exposed to Mo solution. \* $p < 0.05$ , \*\* $p < 0.01$  or \*\*\* $p < 0.001$ .

*NLRP3*, Caspase-1, *ASC* and IL-1 $\beta$  in human primary monocytes, were measured with qPCR. Monocyte cul-

tures, with or without 6 h of priming with *E. coli* LPS (100 ng/mL), were exposed to Co, Cr, Ti and Mo at con-

centrations of 5 and 25  $\mu$ M for 24 h. Neither the exposure to the different metals nor priming with *E. coli* LPS induced any enhanced expression of *NLRP3*, *ASC* or *CASP1* genes (Fig. 3A–C). In contrast, the expression of IL-1 $\beta$  was enhanced after priming with LPS and was further stimulated by exposure to the different metals (Fig. 3D).

### Cytotoxicity and proinflammatory activation in macrophage cultures (THP-1 cells)

The cytotoxicity of Co, Cr, Ti and Mo was examined by adding different concentrations of these metal ions to cultures of phorbol 12-myristate 13-acetate-differentiated THP-1 cells primed for 6 h with *E. coli* LPS (100 ng/mL). After 24 h of exposure, neither Co nor Cr had any effect on viability at concentrations of  $\geq 200$   $\mu$ M, and Ti and Mo caused reduced viability (Fig. 4). However, at concentrations  $\geq 100$   $\mu$ M, Ti completely lacked any cytotoxic effect in this cell-culture assay. An enhanced secretion of IL-1 $\beta$  could be detected in the samples with reduced viability and also at sub-toxic concentrations of Ti (Fig. 4C). IL-1 $\beta$  secretion was reduced in the presence of a caspase-1 inhibitor (Ac-YVAD-cmk; 100  $\mu$ M) with the most pronounced effect observed in the cell cultures exposed to Ti (Fig. 4). Priming of the THP-1 cells with *E. coli* LPS (100 ng/mL for 6 h) before exposure to Ti caused a five-fold increase in IL- $\beta$  secretion, without interfering with the cytotoxic effect of the metal solution (Fig. 5). A statistically significantly ( $p < 0.001$ ) higher secretion of IL-1 $\beta$  was found in cells exposed to both Ti and *E. coli* LPS, and this effect was dose dependent.

### Effect of filtration

The proinflammatory activation caused by Ti in cultures of phorbol 12-myristate 13-acetate-differentiated *E. coli* LPS-primed (100 ng/mL for 6 h) THP-1 cells disappeared after filtration (0.22  $\mu$ m), whereas the cytotoxic effect of the filtered solution persisted (Fig. 6). The solution of



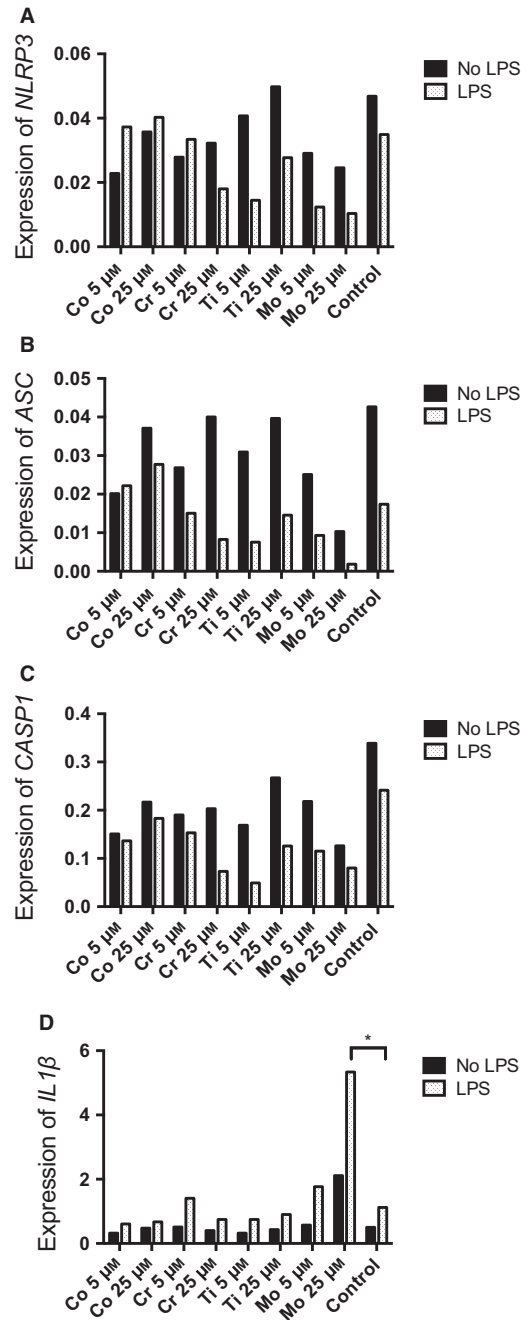


Fig. 3. Gene expression in human macrophages isolated from peripheral blood, with and without *Escherichia coli* lipopolysaccharide (LPS) priming (for 6 h), after exposure for 24 h to cobalt (Co), chromium (Cr), titanium (Ti) or molybdenum (Mo) at concentrations of 5 and 25  $\mu\text{m}$ . Expression of the different NLR family, pyrin domain containing 3 (NLRP3)-associated components was calibrated against that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). (A) *NLRP3* expression. (B) Apoptosis-associated Speck-like Protein Containing a Card (*ASC*) expression. (C) *CASP1* expression. (D) Interleukin IL-1 $\beta$  expression. \* $p < 0.05$ .

400  $\mu\text{m}$  Ti in RPMI 1640 with 10% FBS had lost 94.4% of the Ti when filtered after 24 h, as analyzed using ICP-MS (Fig. 7). This indicates that

the stimulation of IL-1 $\beta$  secretion in cultures of primed THP-1 cells is caused by particles formed in the cell-culture medium, whereas the cytotoxic

effect of Ti is an effect of free soluble ions. Filtration of solutions of the other metals tested (Co, Cr and Mo) did not change the concentration of the metals in these solutions, which indicates that no particle or complex that is unable to pass through a 0.22- $\mu\text{m}$  filter was formed in these solutions (data not shown).

## Analyses of human biopsies and PICF

Gingival mucosal biopsies were taken from three randomly selected patients. Clinical evaluation of the peri-implant mucosa showed that two implant sites were graded as healthy, two sites as mucositis and two sites as peri-implantitis (Table 1). The ICP-MS analyses showed that the Ti concentration in human biopsies varied from 7.3 to 38.9  $\mu\text{m}$ . These levels of Ti in the peri-implant mucosa were within the levels that give a proinflammatory response *in vitro*. PICF samples from the peri-implant pockets showed an IL-1 $\beta$  range from 13.0 to 268.9 pg/mL (Table 1).

*Aggregatibacter actinomycetemcomitans* was detected in three out of six samples from the peri-implant pockets (Table 1). No conclusion could be drawn of the correlations between the concentrations of Ti, the IL-1 $\beta$  level, the presence of *A. actinomycetemcomitans* and clinical data.

## Discussion

This study evaluated the proinflammatory effect in human macrophages exposed to Ti particles formed in cell growth medium, and the synergetic effect achieved when the macrophages were exposed to bacterial components (i.e. *E. coli* LPS) before exposure to Ti. In addition, the amount of Ti found in human tissue surrounding a dental implant was evaluated and compared with the concentrations found to activate and secrete IL-1 $\beta$  *in vitro*.

It was demonstrated that Ti ions form particles in cell growth medium and that the activation and secretion of IL-1 $\beta$  was correlated to particles and not to the soluble ions. Filtration

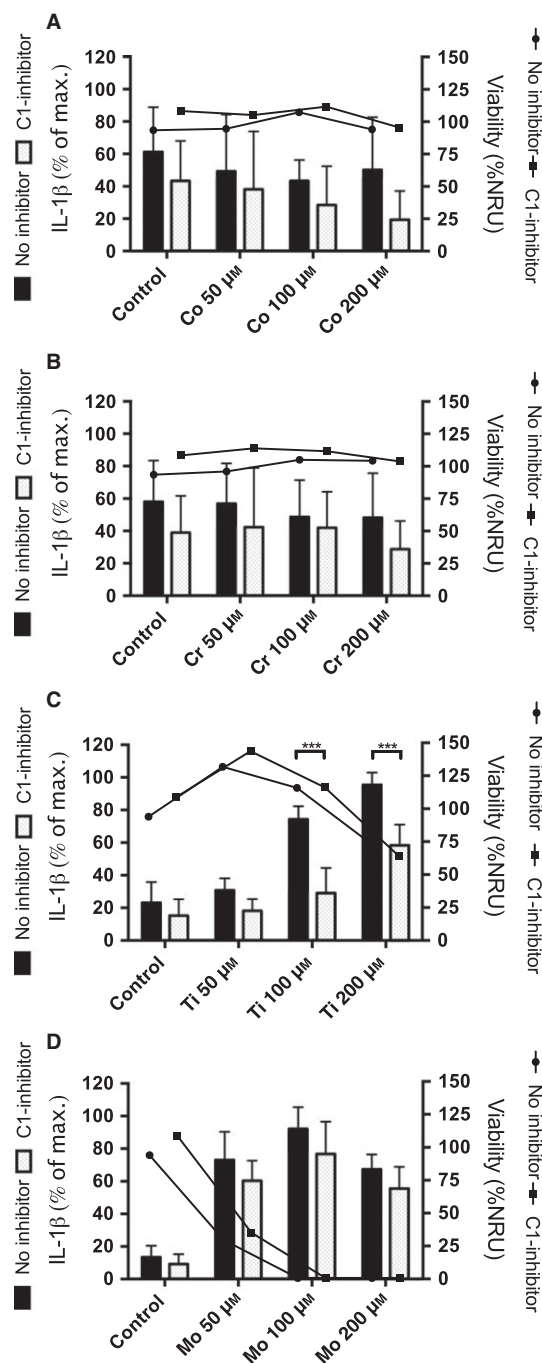


Fig. 4. THP-1 cells primed with *Escherichia coli* lipopolysaccharide (LPS) (100 ng/mL) for 6 h and exposed to cobalt (Co), chromium (Cr), titanium (Ti) or molybdenum (Mo) ions for 24 h. Each bar chart shows release of interleukin (IL)-1 $\beta$  pg/mL, with or without caspase-1 inhibitor. Viability is shown as (%) viable cells in the line chart. (A) Co. (B) Cr. (C) Ti. (D) Mo. \*\*\* $p < 0.001$ .

of the Ti solution abolished its proinflammatory effect on the macrophages, whereas the filtrate retained its cytotoxicity that occurred at higher concentration of ions. This is in contrast to earlier report by Caicedo

*et al.* (70) showing that soluble ions, more than particles, are responsible for the proinflammatory response induced in monocytes/macrophages. Data published by Wachi *et al.* (63) suggest that Ti ions at a concentra-

tion of 9 p.p.m. increase the ratio of RANKL/osteoprotegerin. This confirms our findings that Ti ions increase the secretion of bioactive IL-1 $\beta$  from macrophages, which is a cytokine that stimulates bone resorption through activation of RANKL expression (71). In addition, our results indicate that it is Ti-formed particles, not the ionic form of Ti, as suggested by Wachi *et al.* (63), which stimulate a proinflammatory response. In the present study, a proinflammatory effect could be found also for Mo, but not for Co and Cr, and filtration of Co, Cr and Mo solutions in cell-culture medium did not cause any reduction in the metal concentrations.

This finding, that soluble Mo induces a macrophage proinflammatory response, is in line with that previously reported by Caicedo *et al.* (70).

In the literature it is now accepted that two distinct signals are needed for expression, activation and release of active IL-1 $\beta$ , namely a primary signal and a secondary signal (72). Our results indicate that Ti particles seem to work as a secondary stimulus, activating the inflammasome in the macrophages, resulting in the release of active IL-1 $\beta$  from cells. Different kinds of stimuli (danger-associated molecular patterns) have been found to act as a secondary stimulus and cause inflammation (42,73–75). It has previously been shown that non-degradable TiO<sub>2</sub> nanoparticles phagocytized in pulmonary tissue activate the NLRP3 inflammasome through reactive oxygen species, causing the same inflammation in the lung as seen in patients with asbestosis (76–78). This indicates that the proinflammatory effect caused by the formed Ti particles in the present study is triggered by frustrated phagocytosis, leading to inflammasome activation and release of bioactive IL-1 $\beta$ .

*Escherichia coli* LPS works as a primary stimulus, resulting in an up-regulation in the expression of pro-IL-1 $\beta$  and accumulation of pro-IL-1 $\beta$  intracellularly in macrophages. A secondary stimulus is necessary for the activation and release of mature IL-1 $\beta$ , through the inflammasome-mediated activation

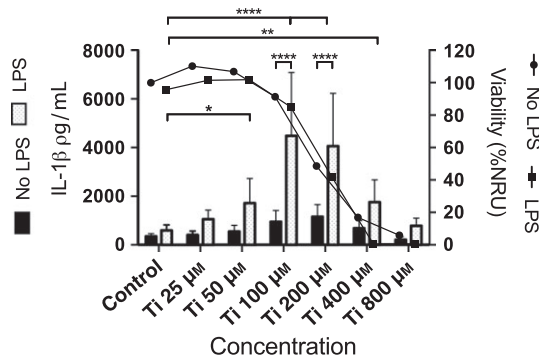


Fig. 5. Interleukin (IL)-1 $\beta$  concentration (pg/ml) in culture medium of THP-1 cells with and without priming with *Escherichia coli* lipopolysaccharide (LPS) (100 ng/mL for 6 h), and exposed for 24 h to titanium (Ti) solution in a concentration range from 0–800  $\mu$ M, is shown. The line chart shows the viability (%) of the cells exposed to the stimulating agent. Values represent mean  $\pm$  SD of three experiments. \*\* $p$  < 0.01 or \*\*\*\* $p$  < 0.0001.

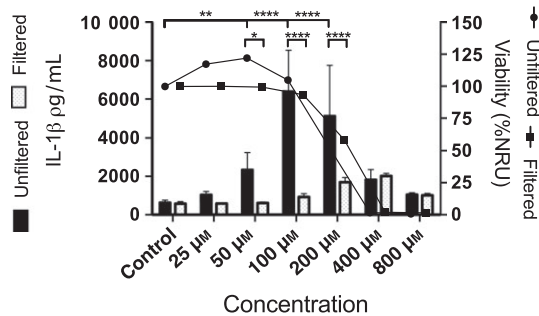


Fig. 6. Secretion of interleukin (IL)-1 $\beta$  from THP-1 cells primed with *Escherichia coli* lipopolysaccharide (LPS) (100 ng/mL) for 6 h and exposed to a filtered or unfiltered solution of titanium (Ti) for 18 h at a concentration range of 0–800  $\mu$ M. Viability (%) is shown in the line chart. \* $p$  < 0.05, \*\* $p$  < 0.01 or \*\*\*\* $p$  < 0.0001.

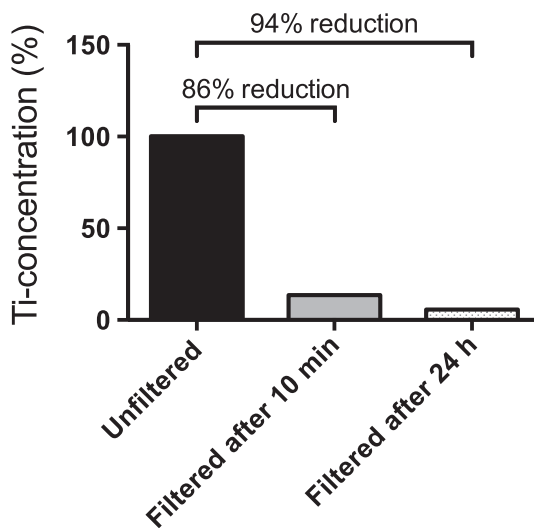


Fig. 7. Reduction of titanium (Ti) concentration in RPMI 1640 with 10% fetal bovine serum (FBS) after filtration through a 0.22- $\mu$ m filter, 10 min and 24 h after addition of the Ti solution to a final concentration of 400  $\mu$ M. The Ti concentration in the unfiltered stock solution was set to 100%. Ti concentration was measured by inductively coupled plasma mass spectrometry (ICP-MS).

of caspase-1 (76–78). We have previously shown that the leukotoxin of *A. actinomycetemcomitans* induces an abundant release of bioactive IL-1 $\beta$  from macrophages that have been prestimulated with bacterial components (57). This is in line with the findings in the present study showing that the effect of Ti alone on IL-1 $\beta$  release from macrophages is negligible compared with that found in macrophage cultures pre-exposed to microbial stimuli.

Quantitative gene-expression analyses of macrophage cultures exposed to sub-toxic concentrations of metal ions showed that the metals, either alone or with pre-incubation with *E. coli* LPS, do not stimulate the expression of mRNA for the inflammasome components *ASC*, *NLRP3* or *CASP1*. In contrast, the expression of pro-IL-1 $\beta$  was enhanced by prestimulation with LPS, and further enhanced by a secondary stimulation with the different metal ions. The lack of effect of the secondary stimulants on gene expression has previously been demonstrated for the periodontitis-associated *A. actinomycetemcomitans* leukotoxin (64). It was also previously shown that *A. actinomycetemcomitans* could up-regulate expression of the NLRP3 inflammasome in human mononuclear cells (60).

IL-1 $\beta$  is a key molecule in tissue remodeling and enhanced levels of this molecule are found in tissue fluids of degenerative diseases, such as periodontitis and peri-implantitis (79,80). The reduced release of active IL-1 $\beta$  in experiments performed in the presence of a caspase-1 inhibitor indicates that the formed Ti particles act through the NLRP3 inflammasome.

Our results show that Ti concentrations found in peri-implant mucosa located in close vicinity to the implants contain similar concentrations of the metal that induces the activation and secretion of IL-1 $\beta$  *in vitro*. The analyses of metal performed on the peri-implant mucosa could not distinguish between particles and soluble ions in the sample. In addition, only three patients were examined in the present study, and further investigations are needed to

Table 1. Qualitative description of samples from three randomly selected patients with a full-arch implant-supported bridge in the maxilla

Patient*	Tissue Ti content <sup>†</sup> (μm)	PICF IL-1β <sup>‡</sup> (pg/sample)	Presence of Aa <sup>§</sup>	Clinical data <sup>§</sup>
Control <sup>¶</sup>	100.1			
1a	7.7	268.9	No	0
1b	14.8	181.0	No	1
2a	8.2	65.5	Yes	2
2b	18.3	114.0	No	2
3a	38.9	26.2	Yes	1
3b	7.3	13.0	Yes	0

\*The three patients are shown as 1, 2 and 3; biopsy site one as 'a'; and biopsy site two as 'b'.<sup>†</sup>Peri-implant crevicular fluid (PICF) levels of interleukin (IL)-1β are given as pg/sample.<sup>‡</sup>Data from the biofilm sample is shown for growth of *Aggregatibacter actinomycetemcomitans* (Aa).<sup>§</sup>0, healthy; 1, peri-implant mucositis; and 2, peri-implantitis (according to clinical examination and intra-oral radiographs).<sup>¶</sup>Titanium (Ti) solution with a concentration of 100 μm is used as the control.

find if there is a correlation between metal content and periodontal inflammation promoting peri-implantitis.

The true cause of peri-implantitis is highly discussed in the dental community. Probably there is a difference between periodontal and peri-implant disease, although clinical and radiological signs of the diseases have much in common. However, there are significant histological discrepancies, which may explain the differences in the onset and progression of the diseases (22,81).

Recent research suggests that bone loss surrounding an implant is not induced by microbes, but is more likely to be a foreign body reaction against the implant (24,82). Our present findings (both *in vitro* and *in vivo*) support both theories of the cause of peri-implantitis. Leaked Ti ions from a dental implant could transform into particles in the surrounding tissue and become phagocytized by macrophages, causing a proinflammatory response, which, in line with our *in vitro* results, is substantially enhanced in the presence of microbial stimuli. If an inflammatory condition is established in the peri-implant mucosa and degradation of bone that supports the implant starts, colonization of bacteria on the implant surface will take place and the process might lead to peri-implant diseases.

In conclusion, we show, for the first time, that Ti ions form particles in physiological conditions that conditions that induce activation and

secretion specifically of IL-1β. Analyses of randomly selected mucosa samples for metal content indicate concentrations of Ti *in vivo* at levels that could initiate activation and secretion of IL-1β *in vitro*. Further studies are needed to determine the role of this finding for the initiation and progress of peri-implantitis.

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